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We previously developed a cell line system in which exogenous expression of estrogen receptor alpha (ERα) in an ERα-negative cell line resulted in ERα -mediated signaling and proliferation. We have now generated cell lines that express ERα only in the cytoplasm (cERa) to characterize the putative cytoplasmic (non-genomic) function of ERα. We have found that the cERα can bind estrogen and is down-regulated, similar to wild-type ERα. Intriguingly, the cERα is completely resistant to ICI182780-mediated degradation. cERα can't activate gene transcription (due to its inability to enter the nucleus), and also can't stimulate cell cycle progression. Consistent with the cERα not activating gene transcription or cell cycle progression, cERα is not able to induce ER-regulated genes. Despite evidence that in some systems that estrogen can have short-term rapid signaling events, we do not find that expression of cERα increases short-term signaling in C4-12 cells. We are currently using a rhodopsin-tagged ER which is in the plasma membrane to determine if membrane association is critical for short-term signaling, and also whether cERα can increase short-term estrogen signaling in MCF-7 cells that overexpress HER2.

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## INTRODUCTION

Since the discovery of membrane estrogen receptor α (ERα) more than 20 years ago, reports on this form of ERα signaling have continued to be documented and have recently received increasing attention. However, this field remains very controversial with nuclear ERα action being studied in much greater detail and becoming much better understood. The IDEA of this proposal is to create a novel and unique model of breast cancer cells that express only cytoplasmic or membrane estrogen receptor (and not nuclear ERα) and then compare and contrast ERα action to cells that express no ERα or wild-type ERα. We are in a unique situation to perform this, as we have recently shown for the first time that ER can be functionally expressed and regulate proliferation in an ERα -ve breast cancer cell line (C4-12).

## BODY

Summary

This progress report is for year 2 of the project. In the first year of the project we made significant progress, having generated stable transfectants that express ERα in the cytoplasm (cERα) and having performed a preliminary characterization of these cells. However, as described in the body of this report, in the second year, we were unable to identify any increase in rapid estrogen signaling in these cells that expressed the cERα. This suggests that either these cells are not suitable for studying rapid estrogen effects or that perhaps the ERα needed to be in or near the plasma membrane to signal in this manner. In the second year we also struggled to create an ER variant that would reside in the plasma membrane. We expect that the small localization tag (myr or CAAX), placed on the N or C-terminus of ERα is folded within the protein and inaccessible for attachment to the plasma membrane. However, we now have a construct, received from a collaborator (Dr Wang, Johns Hopkins University), which is ERα linked to the membrane protein rhodopsin. We show data here that this construct gives membrane localization of ERα and will use this construct in year 3. In year 3 we will also examine the effect of cERα and rho-ERα on rapid estrogen signaling in breast cancer cells that overexpress HER2 and have been shown to exhibit short-term estrogen signaling via MAPK.

**Task 1: To create and characterize ERα-negative MCF-7 cells (C4-12) that stably express GFP tagged membrane ERα (mERα), cytoplasmic ERα (cERα), or wild type (wtERα) (Months 1-12):**

*i) Stably transfect ERα-negative MCF-7 (C4-12) cells with GFP, GFP-wtERα, GFP-mERα and GFP-cERα, and select cell lines that have low and high levels of the receptor (Months 1-6).*

We have stably transfected C4-12 cells with GFP, GFP-wtERα, and GFP-cERα. We have isolated multiple clones and using immunofluoresence microscopy shown that the cERα is indeed expressed in the cytoplasm (compared to wtERa which is mainly nuclear) (Figure 1). GFP alone is expressed all over the cell.

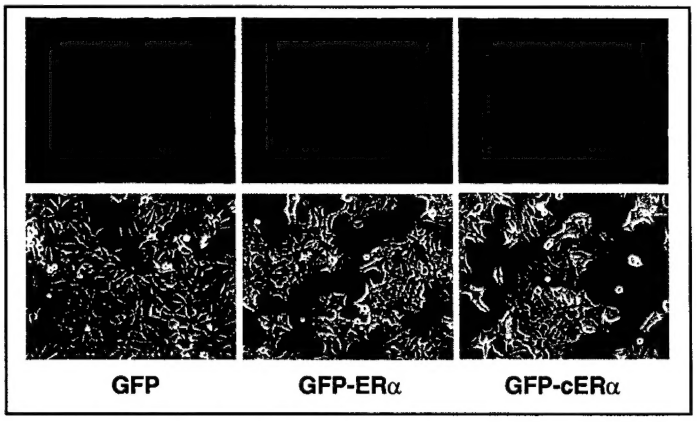


**Figure 1: Stable expression of GFP-cERα in C4-12 cells.** cERα was generated by deletion of the nls (245-270aa) in ERα. Stable clones of GFP, GFP-wtERα, and GFP-cERα were obtained and visualized by fluorescence microscopy (top panels) or by phase contrast (lower panels). GFP was widely distributed over the cell. In contrast, wt-ERα was exclusively nuclear; however, cERα was again widely distributed over the cell and did not show nuclear localization.

We have confirmed that GFP-cERα does not show nuclear localization by biochemical fractionation (data not shown) and confocal microscopy (Figure 2).

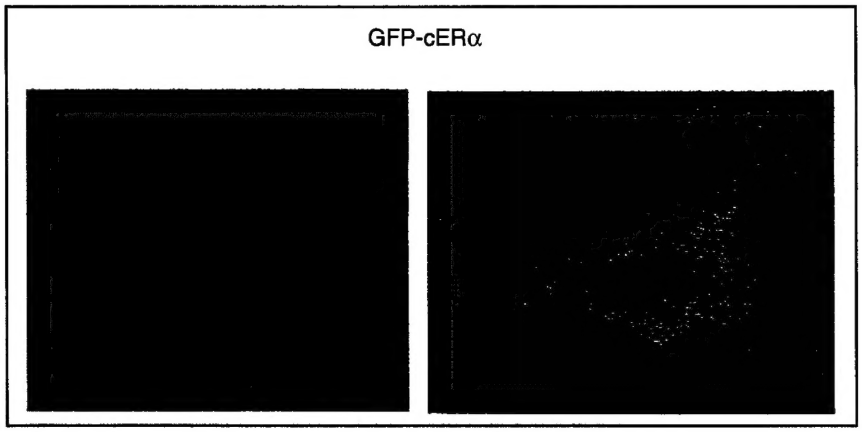


**Figure 2: GFP-cERα is exclusively cytoplasmic.** C4-12 cells stable expressing GFP-cERα were stained with propridium iodide (red) and then examined by confocal microscopy. The GFP signal shows that the cERα is exclusively cytoplasmic.

A setback came however, when we tried to express mERα, which was not targeted to the membrane (data not shown). Tagging of ERα with either C or N-terminal membrane signals does not send ERα to the membrane. This is probably due to folding of the protein making the tag inaccessible. We have therefore entered into collaboration with Dr Wang from Johns Hopkins University. He generated an ERα construct that consists of rhodopsin linked to ERα. Rhodopsin is membrane bound and so directs ERα to the plasma membrane (**Xu Y** *Mol Endocrinol* 2004 Jan, **18**:86-96.) We found the rho-ERα to be in the endoplasmic reticulum following transient transfection with high concentrations of DNA (data not shown), however, lower amounts of DNA caused the rho-ERα to give only a plasma membrane signal (Figure 3).

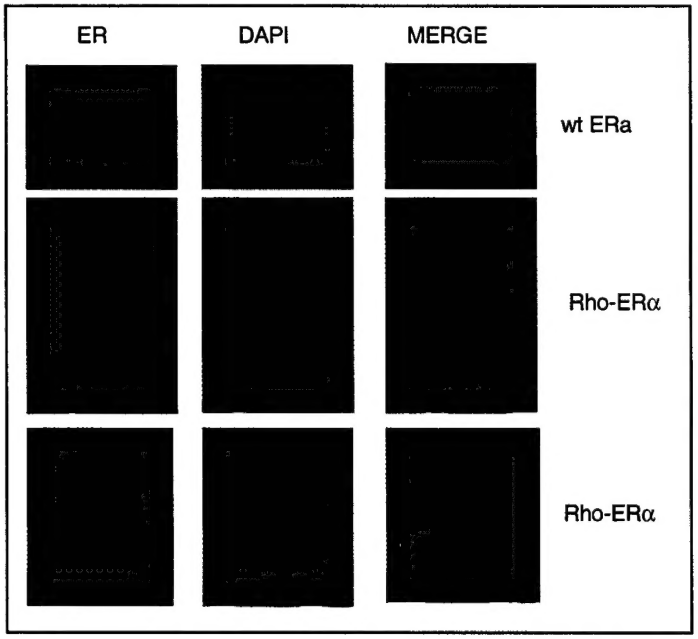


**Figure 3: Localization of rho-ERα.** 293 cells were transiently transfected with wt-ERα or rho-ERα and then ERα detected by immunostaining with ERα antibody (6F11, neomarkers) and detected by anti-mouse antibodies conjugated to alexa488 (left panels). The nucleus was identified by staining with DAPI (middle panels). A merged image is also shown (right panels). As expected, wtERα was entirely nuclear, whereas the rhoERα construct showed exclusive cytoplasmic or membrane staining. We have not confirmed that this is solely membrane staining by confocal microscopy; however, the staining is clearly very different to cERα, and looks very similar to GFP that has a membrane tag on it (data not shown)

Dr Wang has recently published using this rhoERα construct, but all of these studies were performed in HEK293 cells transiently transfected with the rho-ERα. We believe it is important to determine the relevance of these studies in breast cancer cells, and in particular in cells by stable transfection. We are currently therefore stably transfecting this construct into C4-12 cells to see the effect of mERα on rapid estrogen signaling.

*ii) Use biochemical fractionation and confocal microscopy to determine whether mERα and cERα are expressed only in the membrane and cytoplasm respectively, and test whether mERα and cERα are capable of binding estradiol (E2) and tamoxifen (Tam) (Months (4-8).*

Figure 1 confirms that cERα is only expressed in the cytoplasm, while wt-ERα is mainly nuclear. Figure 2 shows that rho-ERα is mainly membranous.

We have not tested directly whether cERα can bind E2 or tam, however an indirect measure is the ability of E2 to downregulate the receptor (which occurs after E2 binding). We found that cERα is degraded following E2 stimulation, suggesting in an indirect way that this receptor can bind E2.

*iii) Examine whether mERα or cERα associate with membrane or cytoplasmic structures (e.g. clathirin-coated pits) (Months 7-12).*

We have not yet performed these assays. These will be done when the rho-ERα stable transfectants are made.

**Task 2: To compare and contrast the effects of estrogen in C4-12-cERα, mERα and wtERα cells (Months 12-24):**

*i) Analyze the effect of short (mins) and long-term (hours) E2 stimulation on localization, movement, and degradation of the different GFP-ERα variants (Months 12-16).*

We have not examined localization and movement, but we have found that the cERα can be degraded by E2 (Figure 4).

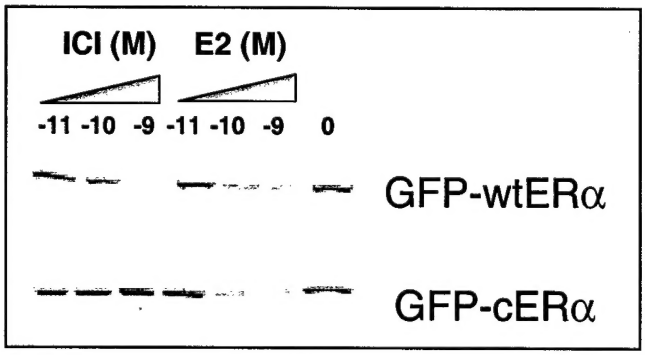


**Figure 4: cERα is completely resistant to antiestrogen ICI 182780-mediated degradation.** C4-12wt-ERα and cERα stable transfectants were starved in serum-free overnight and then treated for 8 hours with increasing concentrations of estradiol (E2) or ICI182780 (ICI). As expected, wtERα protein levels were reduced upon exposure to both E2 and ICI. This effect was blocked with the proteasome inhibitor (lactacystin 10uM) (data not shown). Of note, ICI required a 10-fold higher excess, which has previously been noted by others. In contrast to wtERα, cERα proteins levels decreased upon exposure to E2, but were not affected by any concentration of ICI.

This is an important result given that a number of groups have proposed that E2-mediated degradation of ERα is linked to transcription. cERα is a variant ER that cant activate transcription, thus the degradation of cER by E2 represents a new paradigm for E2-mediated degradation of ER. Interestingly, while wt- ERα is degraded by antiestrogens such as ICI182780, the cERα is not degraded significantly by ICI182780 suggesting that this is a nuclear mediated event and that E2 and ICI degradation mechanisms are distinct. We are interested if membrane localization of rho-ERα will also inhibit degradation. We are currently cloning an SV40 nuclear localization sequence (nls) onto the cERα to put it back into the nucleus and see if it is then able to be degraded by ICI. This will determine whether the loss of ICI-mediated degradation in cERα is due to localization or due to the domain of ERα (240-275aa) that we have deleted.

*ii) Examine whether ER-responsive genes (e.g. TGFα, PgR, cathepsin D, pS2, IRS-1, cyclin D1) are induced by E2 and inhibited by Tam (Months 15-20) by the different GFP-ERα variants.*

We have found that cERα is incapable of inducing expression of genes such as IRS-1, IGF-IR, and cyclin D1 (Figure 5). This is consistent with it not being able to activate gene transcription in an ERE-luc reporter assay (data not shown).

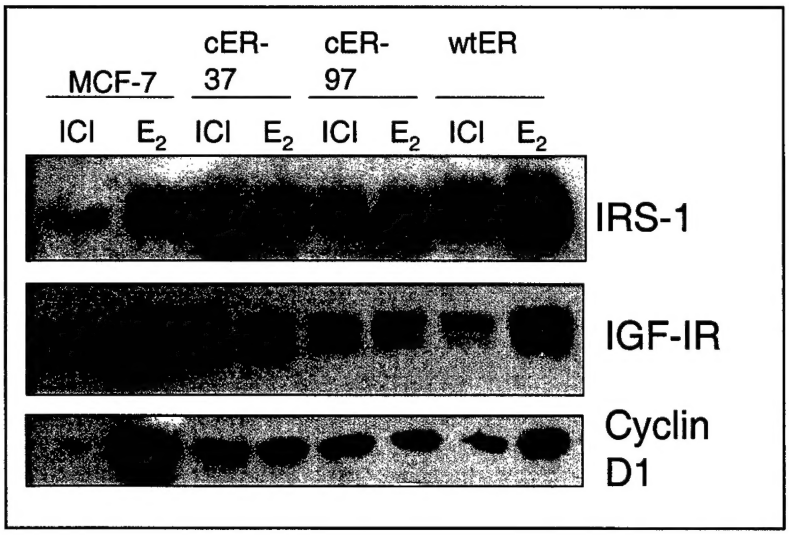


**Figure 5: cERα doesn't confer estrogen-induction of ER-responsive genes.** MCF-7, C4-12cERα and C4-12wtERα cells were starved in serum-free medium overnight and then treated with either antiestrogen (ICI, 10-9M) or estrogen (E2, 10-9M) for 24 hrs. Cells were lysed in 5% SDS and immunoblotted with antibodies to insulin receptor substrate-1 (IRS-1), insulin-like growth factor receptor 1 (IGF-IR) or cyclin D1. As expected, all 3 genes were induced by estrogen in MCF-7 cells and also in C4-12 cells expressing wtERα. In contrast, two stable clones of C4-12ERα did not show estrogen regulation of IRS-1, IGF-IR or cyclin D1.

*iii) Determine whether E2 stimulation results in an increase in S-phase and cell proliferation in C4-12-cERα and mERα compared to C4-12wtERα (Months 18-24).*

We have found that E2 stimulation is able to increase S-phase in wt- ERα cells, but is unable to have an effect in cERα cells (Figure 6), consistent with this variant not inducing gene transcription (Figure 5). This is despite the fact that the cERα can clearly bind E2 and be degraded.

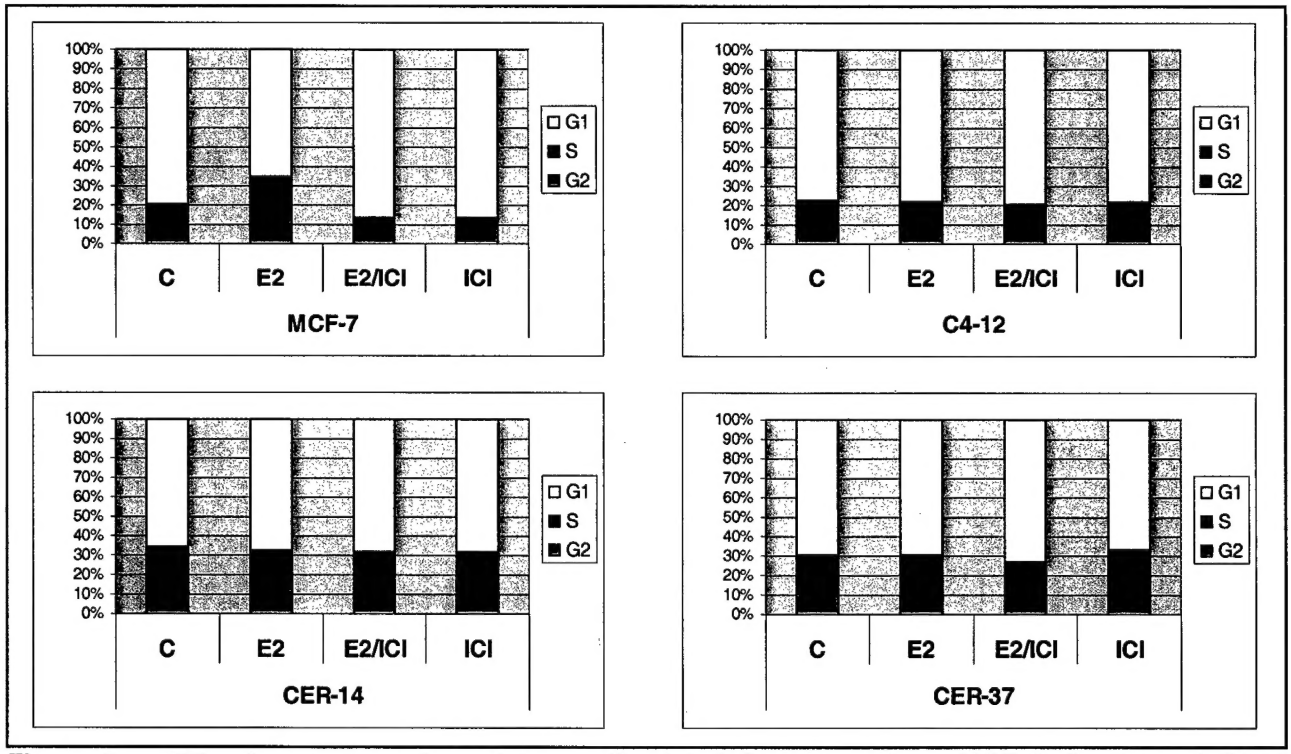


**Figure 6: cERα does not confer estrogen stimulated S-phase entry.** MCF-7, C4-12, and C4-12 cells expressing cERα were starved in serum-free medium overnight, and then stimulated with estradiol (1nm) or ICI (100nM) or the combination for 16hrs. Cells were then fixed in alcohol, stained with propridium iodide and FACS analysis performed. MCF-7 cells stimulated with estradiol showed an induction in S-phase fraction (red) and also an increase in cells entering G2/M (blue). These changes were completely blocked by ICI. In contrast, ERα negative C4-12 cells, or the cERα expressing cells showed no changes in response to E2 or ICI.

**Task 3: To determine whether previously reported short-term (minutes) E2-mediated effects are observed in C4-12-cERα or mERα cells (Months 24-36):**

*i) Perform coimmunoprecipiation and colocalization to determine if mERα and cERα can bind p85 and activate PI3K (Months 24-30).*

Despite preliminary evidence that cERα was able to associate with p85, subsequent experiments failed to confirm an association with either p85 or IGF-IR. In addition we have been unable to show that cER can enhance short-term mediated activation of ERK1/2 or Akt by estradiol (Figure 7).

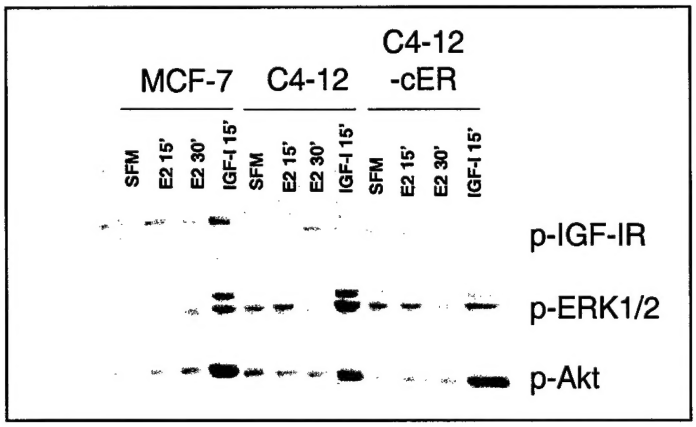


**Figure 7: cERα does not allow short-term estrogen signaling in C4-12 cells.** MCF-7, C4-12 and C4-12cERα cells were starved in SFM overnight and then stimulate with estradiol (1nm, 15 or 30mins) or IGF-I (10nM, 15mins). Cells were lysed and immunoblotted for p-IGF-IR, p-ERK1/2 and p-Akt. MCF-7 cells did not show a response to estradiol at 15 or 30mins. Similar results were also seen in both C4-12 cells. As a positive control, all 3 cell lines responded to IGF-I.

We do not know whether this result simply shows that cERα is not able to activate these pathways in these cells, or whether the specific system we chose is not suitable for detection of short-term estrogen effects. In this regard, members of our group have also recently failed to detect short-term estrogen signaling in MCF-7 cells, but have detected signaling in MCF-7 cells that overexpress HER2 (Figure 8 – kindly provided by Drs Osborne and Schiff, Breast Center, Baylor College of Medicine and in press in JNCI).

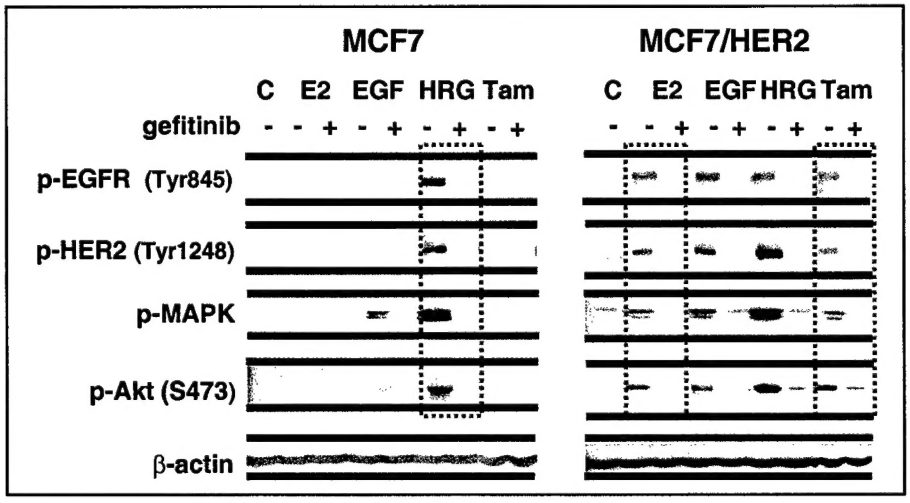


**Figure 8: Rapid estrogen signaling in MCF-7 cells that overexpress HER-2 receptor. MCF-7 and MCF-7 cells stably overexpressing HER-2.** Cells were starved overnight in serum-free medium and then stimulated for 15 minutes with estradiol (1nM), epidermal growth factor (EGF, 100nM), heregulin (HRG, 100nM) or tamoxifen (Tam, 100nM). These incubations were performed in the presence or absence of the EGFR kinase inhibitor Iressa (gefitinib). Cells were then lysed and immunoblotted for phospho-EGFR, HER-2, Akt and ERK1/2 (MAPK). The left panel shows that estradiol or tam is unable to stimulate short-term signaling in MCF-7 cells, which can only be seen with heregulin (red box). In stark contrast, estradiol and tamoxifen were able to activate and phosphorylate all signaling intermediates in MCF-7/HER2 cells, and this was completely inhibited by EGFR blockade. We will therefore use these cells to better understand the role of cERα and rhoERα in this response. Figure kindly provided by Drs Osborne and Schiff, Breast Center, Baylor College of Medicine.

We have therefore decided to test the effect of HER-2 overexpression by two strategies. First we will examine the effect of cERα or rho-ERα overexpression on short-term estrogen action in MCF-7/HER2 cells. Second, we will overexpress HER-2 in C4-12 cells stably expressing the cERα or rho-

ERα. Increased HER-2 will be achieved by retroviral infection using a plasmid from Dr Schiff. In both systems we will then examine short-term estrogen effects

*ii) Examine whether E2-stimulation of C4-12-mERα and cERα cells results in mobilization of intracellular $Ca^{2+}$ and activation of PKC (Months 30-34).*

Not performed yet due to lack of evidence in 3.i. Will be performed if the HER-2 experiments are successful at showing short-term estrogen signaling.

*iii) Determine whether E2 can induce an anti-apoptotic response in C4-12-mERα and cERα cells (Months 32-36).*

Not performed yet due to lack of evidence in 3.i. Will be performed if the HER-2 experiments are successful at showing short-term estrogen signaling.

## KEY RESEARCH ACCOMPLISHMENTS

- Generation of C4-12 cells that express ERα only in the cytoplasm (C4-12- cERα).
- Evidence that cERα is degraded by estrogen but not by antiestrogen

## REPORTABLE OUTCOMES

Development of cell lines:
C4-12-GFP
C4-12-GFP-cERα

## CONCLUSIONS

This project will use a unique cell line model (C4-12) to test if cytoplasmic (cERα) or membrane targeted ERα (mERα) can perform signaling and mediated proliferation. This research is critical, as several recent studies have suggested that cERα or mERα is important, and pathologists only analyze nuclear ERα, which might misclassify a number of breast cancer patients. We have generated cells that express ERα only in the cytoplasm. We find that this receptor can't activate gene transcription or proliferation, despite the fact that the receptor is degraded by E2 and thus presumably can bind E2. However, this receptor is unable to stimulate short term estrogen events. We are currently stably transfecting a membrane variant of ERα (rho-ERα) to see if this can signal. We are also examining the effect of HER-2 overexpression on rapid Estrogen-events. Understanding any potential role of cERα or mERα is critical for the complete understanding of estrogen action and targeting in breast cancer.

## PRESENTATION

2004 "Expression of non-nuclear ER in ER-negative breast cancer cells doesn't confer estrogen-stimulated growth". 14th Annual Breast Cancer Think Tank Meeting, St Kitts.